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THE ORGANIZATION OF THE SUPRACHIASMATIC CIRCADIAN PACEMAKER OF THE RAT AND ITS REGULATION BY NEUROTRANSMITTERS AND MODULATORS

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Our research addresses the cellular organization and regulation of a biological clock that controls daily (circadian) rhythms of behavior (e.g., performance), physiology and metabolism in mammals. This clock, located in the brain's suprachiasmatic nucleus (SCN), can be removed in a slice of hypothalamus, maintained in a life support system for up to 3 days and studied directly. Using this approach, progress in year 2 of this award has been made in: 1) further localizing time-keeping properties within the SCN of rat, 2) identifying electrophysiological properties of neurons in the major SCN subdivisions, 3) establishing regulatory roles for serotonin, a neuromodulatory input from the brain's arousal center in the raphe nuclei, as well as for neuropeptide Y, an input from the intergeniculate area, 4) determining the level of glutamic acid decarboxylase (GAD), the biosynthetic enzyme for the inhibitory neurotransmitter GABA, in SCN over the circadian cycle, and 5) examining the potential roles of excitatory and inhibitory amino acids in regulation of the SCN by retinal inputs carried by the optic nerve. This project involves both individual and interactive research projects at the University of Illinois and the USAF School of Aerospace Medicine.

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RESEARCH OBJECTIVES

In mammals, the suprachiasmatic nucleus (SCN) of the hypothalamus is a circadian pacemaker that serves a well-defined, critical role in the generation and entrainment of daily rhythms of physiological, metabolic and behavioral functions. The SCN contain an endogenous pacemaker that generates near 24-hr SCN rhythms of electrical activity and vasopressin secretion. Outputs from this central pacemaker time cellular, tissue and organismic circadian rhythms. All circadian rhythms are reset by changes in environmental lighting, which can affect the SCN through inputs from the retina, intergeniculate leaflet or the raphe. However, little is known about the way in which the neuronal components of the SCN are organized to carry out time-keeping or to analyze phase-resetting information. This study seeks to determine 1) the functional organization of the SCN by electrophysiological analyses of regional distribution of pacemaking properties and neuronal characteristics as well as 2) SCN responses to extrinsic and intrinsic neurotransmitters and modulators.

We are using the rat hypothalamic brain slice to study the functional organization of the SCN directly. Our work has established that circadian pacemaking and resetting properties are endogenous to the SCN and can be studied *in vitro*. In the studies undertaken in year 2 of this award, the circadian rhythm of SCN electrical activity was recorded continuously in intact and microdissected slices of rat hypothalamus for 32 hr after slice preparation. Persistence of a rhythm in microdissected subregions was determined. Whole cell recording in slice of single SCN neurons was performed over the circadian cycle to assess the range of electrophysiological characteristics in each SCN region together with diurnal changes in electrical properties. The neuromodulators serotonin and neuropeptide Y were applied focally with micropipette, and effects on the phasing of the electrical activity rhythm determined for 24-48 hr after treatment. Additionally, the levels of glutamic acid decarboxylase (GAD), the biosynthetic enzyme for the inhibitory neurotransmitter GABA, and nitric oxide synthase (NOS), a possible mediator of glutamate stimulation, were assessed over the circadian cycle. In experiments directed at understanding regulation by retinohypothalamic afferents, Dr. Rea's lab at the USAF-SAM has examined release of excitatory amino acids, field potential activity and pharmacological blockade in SCN upon stimulation of the optic nerve.

The main hypotheses tested in this study include: 1) pacemaking properties are distributed throughout the SCN; 2) the neurons of the SCN are homogeneous with respect to their electrical and pacemaking properties; 3) neuromodulators from inputs implicated in phase-shifts of behavior by dark pulses (serotonin from the raphe, neuropeptide Y from the intergeniculate) are effective phase-shifting agents for SCN during the circadian day; 4) GAD levels are constant over the circadian cycle; and 5) light information carried by the retinohypothalamic tract affects the SCN via excitatory amino acids (*viz.*, glutamate).

The long-term goal of these studies is to understand how neurons of the SCN are organized to generate a 24-hr biological clock and what role specific neurotransmitters and modulators play in the pacemaking and resetting process. Because the SCN integrate most circadian behaviors and metabolic fluxes, this study has basic relevance to understanding circadian dysfunction induced by transmeridian travel and sustained, irregular work schedules, with application to improving human performance under conditions that induce circadian desynchronization.

PROGRESS TOWARD SPECIFIC AIMS:

The following specific aims, formulated in terms of hypotheses to be tested, have been addressed in the second year of the award. Most are stated in the original proposal and some have developed from the findings made in Year 1. Substantial progress has been made toward each. A summary of the rationale of the experiments, the methodological approach, the results and the interpretation of each follows.

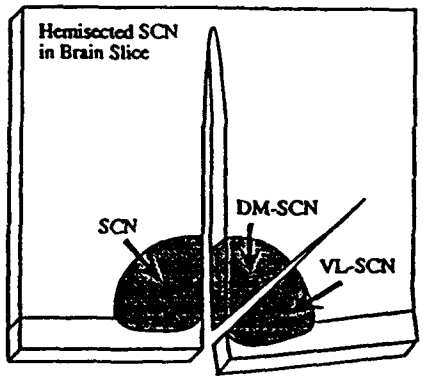


FIG. 1. SCN BRAIN SLICE AND MICRODISSECTION COORDINATES FOR SUBDIVISION EXPERIMENTS.

1) PACEMAKING PROPERTIES ARE DISTRIBUTED THROUGHOUT THE SCN. This hypothesis has been tested by microdissecting the SCN into the well described dorsomedial (DM, source of efferents) and ventrolateral (VL, region that receives afferents) subregions (Fig. 1) and measuring the ability of each part to generate a circadian rhythm of neuronal activity. Activity is compared with that in the same subregions of intact SCN as well as in subdivided SCN in which only one region remains. Concurrently, a second approach, that of whole cell patch recording, has been developed this year to examine properties of individual neurons, some of which may contribute to pacemaking; this is developed in section 2.

We have found that both the VM and DL regions oscillate in the intact SCN, as well as in single SCN whose connections to the other member of the bilateral pair in the brain slice have been severed by cutting ventral to the third ventricle which bisects the slice. Subsequent experiments have assessed the firing pattern after bisection of the slice and then hemisection of the SCN into DM and VL regions, or in the absence of one of the two subregions. Measurements were made on these regions over 32 hr.

METHODS

Hypothalamic brain slices containing 500 μm coronal sections of the paired SCN were prepared and hemisected; in some experiments either all VL or all DM subregions were discarded. Control slices were left intact. Dissection involved bisected the slice by cutting the fibers connecting the paired SCN, isolating each nucleus from the other, then hemisecting the SCN by cutting at a 45° angle from the base of the 3rd ventricle; this effectively divides DM and VL regions. One hr after dissection at CT 1,

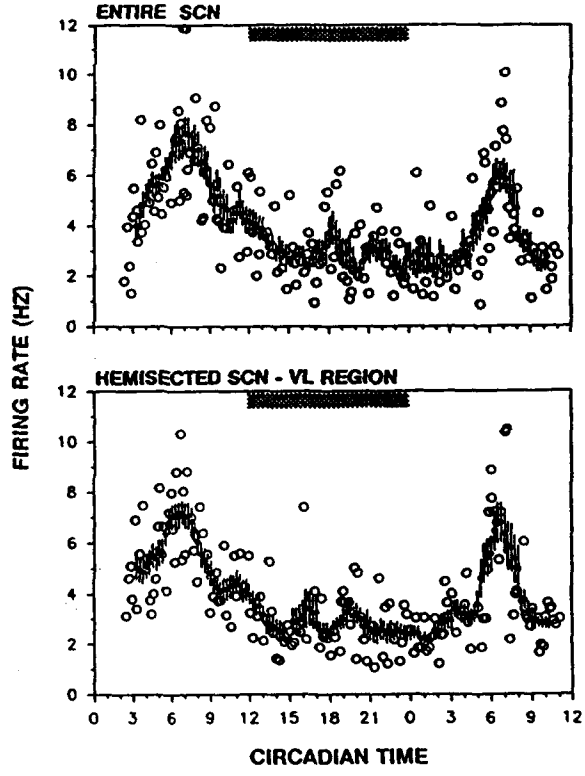


FIG. 2. RHYTHM OF ELECTRICAL ACTIVITY IN ENTIRE SCN VS VL SUBDIVISION

isolated regions were examined over the next 32-hr for evidence of an electrical circadian rhythm.

BRAIN SLICE. Coronal brain slices containing a 500 μm section of the SCN are prepared during the day from 2-3 month old Long-Evans rats housed on a 12:12 LD (light/dark) cycle. Slices are perfused in a brain slice chamber and situated at the liquid-gas interface. The slices are perfused with a minimal medium containing Earle's Balanced Salt Solution, supplemented with 24.6 mM glucose and 26.2 mM $NaHCO_3$, saturated with 95%O_2/5%CO_2, and maintained at 37°C and a pH of 7.40. The slices remain illuminated throughout an experiment.

ELECTRICAL RECORDING. Average firing frequencies for individual neurons are used to gain evidence for a circadian rhythm of electrical activity. Spontaneous neuronal firing of single neurons is recorded extracellularly. Individual neurons are identified by their firing pattern and action potential waveform and an average firing rate is calculated over a minimum of two two-minute periods. This procedure is repeated for as many cells as possible for the duration of a recording session, usually 12 or 24 hours. Recording sites are arbitrarily chosen to reflect a random sample of neurons within the isolated region being studied.

DATA ANALYSIS. Circadian phase of the SCN can be determined by empirical analysis of sliding window averages. Raw data from individual cells are grouped into two hour bins, incremented in 15 minute steps, from which average firing rates and standard errors are calculated. This treatment acts as a low-pass filter, smoothing out high-frequency variability in the raw data and preserving the low-frequency oscillation. The phase of the electrical rhythm is determined by visually estimating the time-of-peak from the sliding window averages plotted against circadian time. The normal time-of-peak is CT 6.9, or 6.9 hours after the lights are turned on in the colony.

RESULTS

Empirical analysis demonstrates that a robust oscillation can be generated by the VL-SCN, even when surgically deafferented from the DM region (Fig. 2). Most surprisingly, this rhythm is indistguishable in detail from that in control slices. The DM region, however, generally exhibited degradation of the electrical rhythm after hemisection (Fig. 3a, b, c.) Those cases in which high amplitude rhythmicity was retained in DM subdivisions, the cut dividing the SCN was biased ventrolaterally (Fig. 3c). When activity was assessed in experiments where only VL or DM regions remained in the dish, to control for diffusible factors that might sustain rhythmicity, the VL region still produced an apparently normal, high amplitude rhythm (Fig 4, next page.)

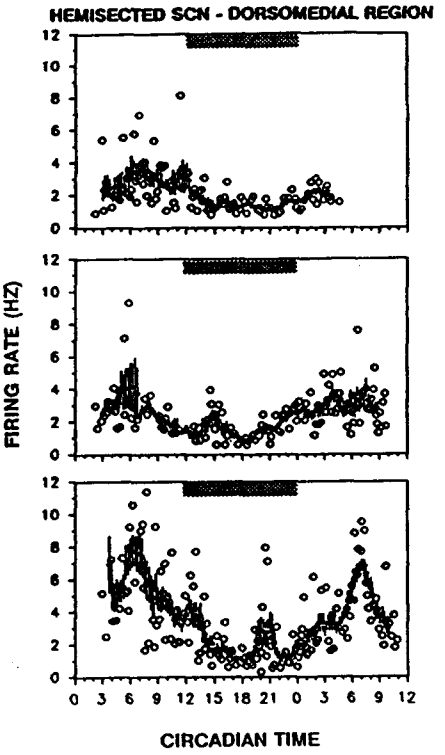


FIG. 3. RANGE OF CIRCADIAN PATTERNS OF POPULATION ELECTRICAL ACTIVITY IN DM REGION; LOWEST PANEL = VL-BIASED CUT.

DISCUSSION

These findings demonstrate that coronal slices of the VL region of the SCN contain an autonomous near 24-hr pacemaker. The circadian rhythm in electrical activity generated by this piece of tissue, which contains considerably less that 1/3 of the total SCN, is indistinguishable from oscillations of intact SCN, both in period and amplitude. The rhythm persists in the absence of the DM region. Thus, the cells in a slice of the VL region contain sufficient information to generate circadian rhythmic properties of the SCN. The presence of a functional pacemaker in the isolated DM-SCN is questionable. It's electrical activity is very different from a normal circadian rhythm, but it does appear to have some pattern. A high amplitude circadian pattern emerges as more of the central SCN in included. One can conclude from this research that integration of various neural assemblies within the SCN are necessary for normal pacemaker function throughout. The essential components of the pacemaker appear to be primarily localized in the VL-SCN.

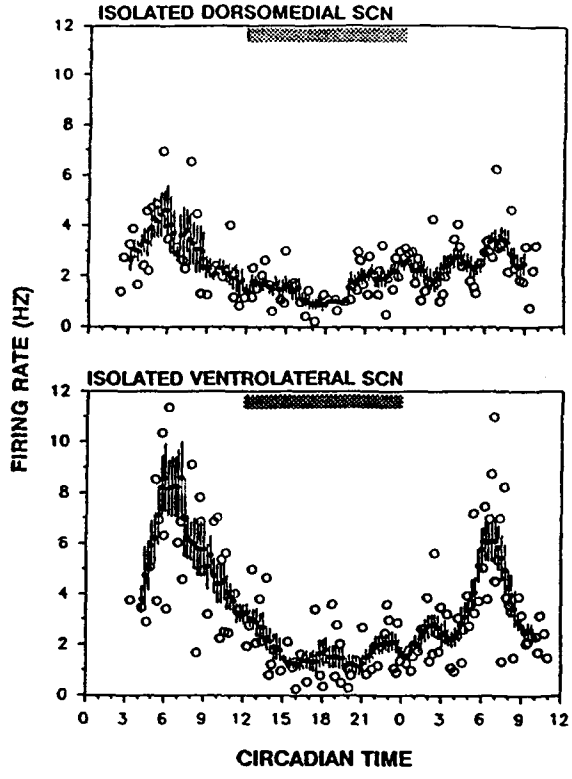


FIG. 4. CIRCADIAN PATTERNS OF ELECTRICAL ACTIVITY IN ISOLATED DM- OR VL-SCN.

FUTURE RESEARCH

The primary goal of this research has been completed: we have clearly demonstrated that a pacemaker exists in the VL SCN, and its absence in the DM region cannot be disproved. We will now move in two directions to further understand functional organization. The first is to examine properties of individual neurons in each subregion to understand how they generate the functional properties that we have thus far measured in an integrated way (see next section on whole cell recording in the SCN brain slice.) The second project will employ optical recording techniques to study dynamic relationships within the SCN. This project will attempt to relate multiple cell activity to the neuroanatomical and immunohistochemical organization of the SCN. This integrated analysis to be performed by Tom Tcheng, a Neuroscience Program graduate student, will provide further insight into pacemaker organization.

2) SCN NEURONS ARE HOMOGENEOUS IN THEIR ELECTRICAL AND PACEMAKING PROPERTIES.
Initial work, discussed in the annual report for year 1, and significantly extended this year (see above), has demonstrated potential differences in the ability of DM and VL SCN to produce a robust circadian rhythm of neuronal activity. This could represent differences in the extent to which the 'clock' mechanism is present in each region. It could also be indicative of differences in the populations of neurons within each region, be they part of the clock or strictly output elements.

In order to address the issue of regional differences at the cellular level, we have begun to examine the individual neurons of the SCN, using the whole cell recording technique in rat brain slice. Our initial efforts were concentrated in the VL-SCN, although we have recently begun to extend our observations to DM-SCN. The VL-SCN has proved to be composed of a variety of electrophysiologically distinct cell types. This disproves the above hypothesis, which had been suggested by early workers in the field, such as Thompson and Wheal (*Neuroscience*, 1974.) Furthermore, preliminary findings concerning DM-SCN neurons do not contradict the alternative hypothesis that there *are* regional differences in properties of neurons of the VL and the DM-SCN. This technique was brought to the lab and first applied to the SCN by Dr. Eve Gallman, a postdoctoral fellow; she conducted experiments described in this section.

METHODS

Preparation of brain slices

Coronal hypothalamic slices were prepared from Long-Evans rats 6-9 weeks old. Rats were from our inbred colony, were maintained on strictly controlled lighting schedules, and were on a given lighting schedule for at least 3 weeks at time of sacrifice. Animals were sacrificed by rapid decapitation (guillotine), the brain quickly removed, the sides and dorsal aspect blocked off, and the remaining tissue placed on the sled of a guillotine-style tissue chopper. Slices were cut at 500 μm thickness and immediately transferred to a dish of medium (GIBCO EBSS, supplemented with $NaHCO_3$ to 26mM and glucose to 10mM). From each brain, 1 or 2 slices were obtained which contain the paired SCN, visible as dark regions located ventrally on either side of the 3rd ventricle. These slice were transferred to a perifusion-interface chamber where they were maintained at 37°C with an outer bath bubbled with 95% O_2/5% CO_2. All slicing was done during the donor rat's subjective day (*i.e.*, between CT 0 and CT 12; CT = circadian time, starting at the onset of light in the daily cycle of the rat colony) as slicing at this time does not reset the circadian rhythm [Gillette, Brain Res., 1986]. Slices were maintained for periods of up to 48 hours.

Electrophysiologic recording

Recordings were accomplished using the whole cell recording technique of Blanton, *et al.* [J.Neurosci.Methods, 1989] which was adapted from the original patch clamp protocol of Hamill, *et al.* [Pflügers Arch., 1981]. Patch pipettes were pulled from capillary glass on a Flaming/Brown model P-87 micropipette puller to yield tip size of 2.0 to 2.5 μm and tip resistance 5-10 megaOhm and were filled with electrolyte consisting of (in mM): 130 K^+gluconate, 10 EGTA, 10 HEPES, 1 $CaCl_2$, 2 $MgCl_2$, 1 Na^+ATP. Each micropipette, held in a microelectrode holder equipped with a pressure port (WPI MEH2SW) connected to an Axon Instruments HS-2 headstage/preamplifier (gain 0.1x), was placed on the surface of an SCN, as visualized under a dissecting microscope. The pipette was advanced under the control of a Narishige hydraulic microdrive (MO-8 or MW-4). When a cell was encountered and a seal formed between the pipette and the cell, the underlying membrane was generally ruptured with light suction. On some occasions, the ionophore, nystatin, was included in the pipette and the membrane permabilized rather than being ruptured. Some stimulation protocols and most analysis were performed using the software package, pClamp (Axon Intruments).

General Protocol

Recordings were generally restricted to neurons in the ventolateral aspect of SCN. Passive properties of a neuron were determined in current clamp mode by use of repeated hyperpolarizing and depolarizing pulses of minimum 200 msec duration. From the voltage responses to these pulses, input resistance was determined. A range of pulse amplitudes were used in order to detect rectifying currents. Pulses were long enough to detect delayed onset, voltate dependent currents. Membrane time constants were determined from the charging phase of a hyperpolarizing pulse within the linear response range of the cell. Membrane conductance was estimated from the time constant, assuming a membrane capacitance of 0.01 $pF/\mu m^2$.

A twenty second depolarizing pulse was used to determine basic firing characteristics of a neuron. In spontaneously firing neurons, action potential (AP) amplitude, width at half maximum amplitude, and amplitude of afterhyperpolarization or afterdepolarization, if detected, were measured. Non-firing cells were induced to fire either: a) on rebound from a hyperpolarizing pulse, or b) by direct depolarization to threshold.

The location of each neuron and the CT when it was studied were recorded. Data were pooled, by time of acquisition, into six four hour bins or 12 two hour bins.

RESULTS

Intrinsic membrane properties

Fifty-nine neurons have been studied to date. Length of recording varied from 3 to 240 minutes, average 47 min. The major criteria for acceptance were a stable recording and a healthy action potential as judged by amplitude, rate of rise, and duration . The resting membrane potential values were viewed with caution (see below). However, only cells with apparently zero-crossing action potentials were accepted.

Neurons studied in the whole cell patch configuration are subject to 'rundown'. Therefore, all data concerning intrinsic properties were obtained within the first 15 minutes of recording. In cells held for longer periods of time, additional determinations of these properties were made and compared to the earlier determinations. While not all cells showed signs of rundown or deterioration over time, those that did generally began to deteriorate after 30 minutes of recording.

TABLE I: Basic electrical properties of SCN neurons

	Mean ± s.d.	(n)	Range
Resting potential (mV)	-50.5 ± 6.4	(56)	-40 to -67
Input resistance (MΩ)	577.9 ± 241.6	(48)	108 to 1100
Membrane time constant (msec)	22.2 ± 9.5	(47)	1.8 to 51.7
Conductance ($\mu S/cm^2$)	61.6 ± 73.9	(47)	19 to 544
Action potential:			
Amplitude (mV)	64.1 ± 11.5	(55)	40 to 100
Half-peak duration (msec)	0.84 ± 0.22	(47)	0.4 to 1.6
Firing threshold (mV)	-54.0 ± 6.3	(34)	-42 to -69
Firing Frequency (Hz)	4.4 ± 4.5	(51)	0 to 18

Circadian variation in neuronal properties

Roughly 60 neurons have been studied across the circadian cycle, with between 5 and 13 neurons per four hour time bin. The mean resting membrane potential of this sample showed circadian variation, being somewhat more negative during subjective night than during subjective day (Fig. 5). The actual transition to the more negative potential preceded the day-to-night transition.

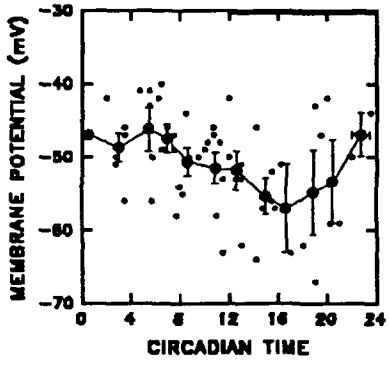


FIG. 5

The technique of whole cell patch, as it is considerably more invasive than an external recording, might be expected to affect the spontaneous firing rate. Nevertheless, as shown in Fig. 6, the same general pattern, a higher firing rate during subjective day and a lower rate at night, was seen. Further, while about one quarter of cells studied during subjective day fired below 0.5 Hz or were silent, fully half of those studied at night fell into this group.

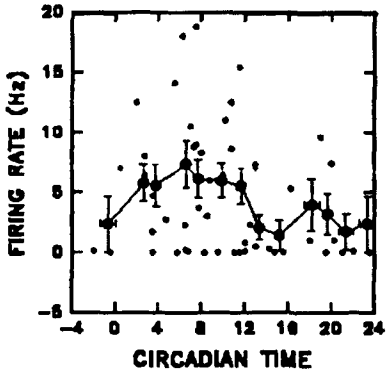


FIG. 6

Resting membrane potential - a caveat

Unlike an intracellular impalement, which offers a high resistance pathway for exchange of substances between electrode and neuron, a whole cell patch, with a 2-3 μm pipette tip sealed to a 10-15 μm cell soma, allows for rapid exchange of pipette and cytoplasm. While advantageous in some respects, this circumstance plays havoc with measures of cell resting membrane potential (RMP). In addition to the offset often found when an electrode or pipette is withdrawn from a neuron, one must estimate and correct for an offset due to the tip potential. Further, as the cell contents are rapidly adjusted, by diffusion, to reflect pipette contents, any changes in concentration of major permeant ion species will affect the resting membrane potential. Thus values reported here for RMP are estimates only. While these values may be in error, this error should either be completely random, or possibly, overestimate of most potentials (*e.g.* a value of -40 mV may in fact have been -48 mV). This second type error is suggested by intracellular work which puts mean RMP near -59 mV [Wheal & Thomson, *Neuroscience*, 1984]. It is unlikely that any such error could account for the general trends seen across the circadian cycle and we believe such trends to be real.

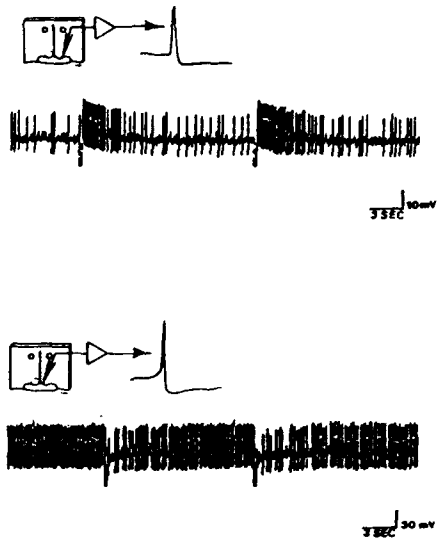


FIG. 7

Evidence for heterogeneity of neuronal cell types

A number of characteristics were noted in some, but not all neurons studied. For instance, many cells displayed a depolarizing sag during extended hyperpolarization. Other neurons had no indication of a delayed onset current of this type. Most neurons showed some form of post-inhibitory rebound (PIR) excitation. Some responded to a hyperpolarizing pulse with a burst of action potentials lasting up to 10 seconds. In a subset of neurons with PIR, the burst rode on a strong wave of depolarization suggestive of the low-threshold calcium current (Fig. 7A). This current has been recently reported in SCN [Kim & Dudek, *Neurosci. Abstr*, 1991]. In marked constrast, a few neurons responded to release from a hyperpolarizing current pulse with a period of inhibition (Fig. 7B). None of the neurons responding with transient inhibition were found in the VL-SCN. It is possible that this represents a regional variation in the neuronal population of SCN.

In response to a 20 second depolarizing pulse, most neurons showed little spike frequency adaptation (SFA) within the first second. The steady state firing rate at the end of the 20 second stimulation was generally 30 to 50% of the rate during the first half second. Figure 8A presents a typical VL-SCN neuron with this response. The DM-SCN neuron in Figure 8B, however,

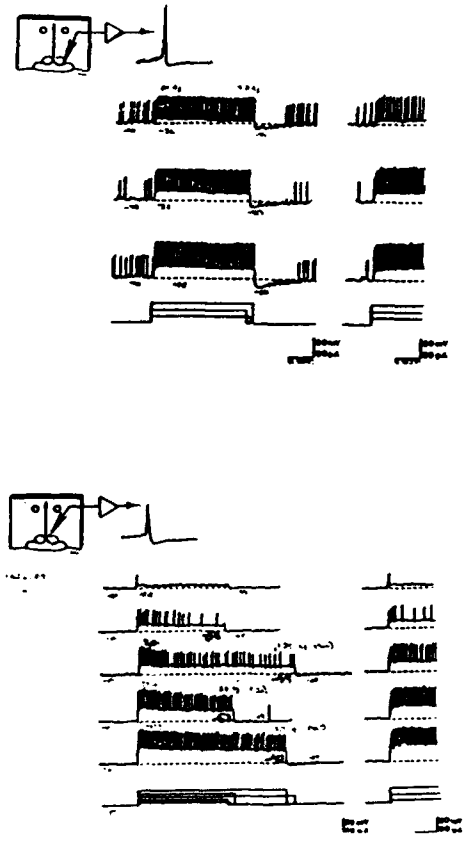


FIG. 8

demonstrates both initial SFA and a dramatic 90% reduction in firing rate from the initial to the eventual steady state rate. This was the greatest degree of SFA seen in our SCN neurons. This difference in repetitive firing properties may reflect an underlying difference in the way DM and VL-SCN neurons integrate and pass on excitatory input.

SUMMARY

There is ample evidence that the SCN must be considered to be at least as neurally complex and varied as any hypothalamic area previously studied. Our evidence suggests that there may be underlying differences in the manner in which the activity of these neurons is modulated by excitation and inhibition.

FURTHER DIRECTION

Many more neurons must be studied, especially from the DM-SCN, before any firm conclusions can be made concerning regional differences in electrophysiologic characteristics. Pharmacological intervention and ion substitution experiments must be done to confirm the identity of the currents modulation the firing properties noted above. This information may give clues as to how the clock is able to regulate the neural output of a subpopulaiton of SCN neurons to yield the well-documented circadian rhythm of ensemble activity of this region.

There are regional differences in the localization of neuromodulators within rat SCN. For instance, vasoactive intestinal peptide-containing cells are generally found in ventral and VL-SCN, while somatostatin-immunoreactive cells are located more dorsally, and vasopressin cells even more dorsally, in DM-SCN.

Combined electrical and immunochemical characterization of individual neurons will aid in understanding the roles of the various cell types in shaping the output of the SCN.

3) SEROTONIN, A NEUROMODULATOR CONTAINED IN AFFERENTS FROM THE RAPHE, AND NEUROPEPTIDE Y, A NEUROMODULATOR IN PROJECTIONS FROM THE INTERGENICULATE LEAFLET, ARE EFFECTIVE PHASE-SHIFTING AGENTS DURING THE CIRCADIAN DAY. This hypothesis was tested by evaluating the effects upon the rhythms of neuronal activity of focal application of a 30 *fl* droplet of 10^{-6} M serotonin or neuropeptide Y to the SCN region receiving these inputs. We also have evaluated the permanence, specificity, dose-dependency and G-protein coupling of the serotonin effect. These experiments are begin conducted by Marija Medanic, a Physiology & Biophysics graduate student in our laboratory.

METHODS

Rat SCN were isolated in a 500 μm hypothalamic brain slice and maintained in a Hatton-style (Hatton, 1980) brain slice chamber for up to three days. The slices were continuously supplied with 95% O_2, 5% CO_2 and perfused with glucose and bicarbonate supplemented Earle's Balanced Salt Solution (GIBCO). The slices were treated on day 1 by localized applications of serotonin and serotonin-specific agonists (8-OH dipropylaminotetralin (8-OH DPAT) and 5-carboxamidotryptamine (5-CT, RBI; Sanders-Bush, 1988) or neuropeptide Y (NPY, Sigma) to the ventrolateral regions of one of the SCN in the slice. A microelectrode filled with a pharmacological agent (10^{-6}M) was positioned over the desired region of the slice and a spherical drop of 10^{-11}ml was deposited on the region of the SCN that receives afferent fibers. In separate experiments, slices were treated at

different time points across the circadian cycle (circadian time 0, CT 0, being at the onset of light and continuing for 24 hours). Specific serotonin agonists were applied at CT 9, a time at which serotonin was known to affect the pacemaker, to determine the specificity and receptor subtype of the serotonin-induced phase-shifts. The effects of the treatments on the phase of the pacemaker were assessed on the second and third days *in vitro* by extracellularly recording the rhythm of neuronal activity, and comparing the time-of-peak to that of control slices treated with medium droplets. Normally, the time-of-peak between experiments is highly stable and predictable so that the peak can be used as a marker of the phase of the circadian pacemaker (Prosser & Gillette, *J. Neuroscience,* 1989).

RESULTS

The following summarizes our understanding of action of serotonin on the SCN prior to this year:

1. *Serotonin effects the SCN pacemaker directly*, inducing the largest phase-advances (6.9 $\pm$ 0.1 hr) at CT 7.

2. *Serotonin permanently resets the phase of the oscillator.* Administration of serotonin at CT 7 on day 1 resulted in a 6.9 hr phase-advance of the neuronal activity rhythm recorded on day 2; the same magnitude advance was maintained on day 3.

3. *Serotonin effects the phase of the SCN in the daytime.* Treatment of SCN slices in the daytime resulted in significant advances in the phase of the rhythm of neuronal activity, with the greatest sensitivity at CT 7, whereas treatment at night did not (see Fig 10.)

4. *The change in the phase of the pacemaker is serotonin-specific.* The specificity of the phase change induced by serotonin was assessed by treating slices with 5-CT, an agonist specific for the 5-HT_1 receptor subtype as well as 8-OH DPAT, specific for 5-HT_{1A} receptor subtype. Application of 5-CT at CT 9 resulted in a 6.5 $\pm$ 0.2 hr (n=3) advance where at CT 15 (n=2), a point when the SCN is insensitive to serotonin, it caused no effect on the time-of-peak in the next oscillation. Application of 8-OH DPAT at CT 9 induced 7 $\pm$ 0.1hr (n=3) phase advances.

During the current year we have made progress in the following aspects of this study:

1. The effect of serotonin on the SCN pacemaker is dose-dependent. The effects of serotonin at different concentration (ranging from 10^{-5} M to 10^{-10}M 5-HT) at CT 9 were assessed on the second day *in vitro* (Fig. 9.) The phase-shift was dose-dependent between 10^{-7} and 10^{-11} M, with a half maximal shift induced at 10^{-9} M serotonin.

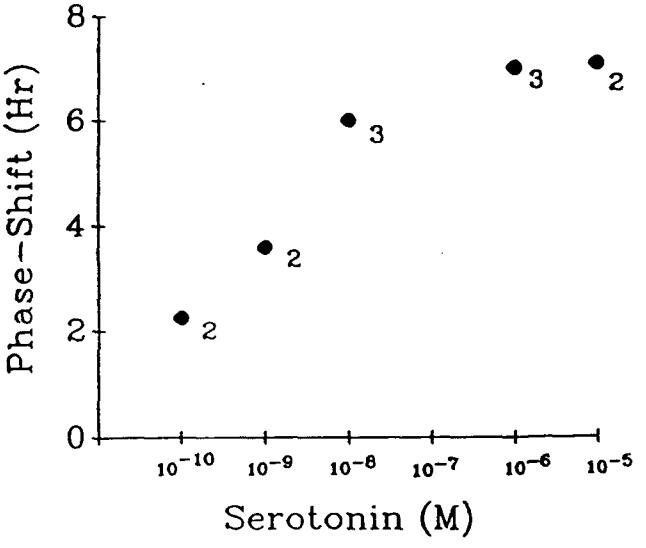


FIG. 9. DOSE-RESPONSE CURVE FOR PHASE-SHIFTS INDUCED BY MICRODROPS OF SEROTONIN. CIRCLES = MEAN ± SEM PHASE-SHIFTS. N=NUMBER OF EXPERIMENTS.

2. Serotonin-induced phase shifts are mediated by a PTX-sensitive G protein pathway. Pretreatment of the SCN slices with a 6-hr bath application of a pertussis toxin (PTX) was found to block the phase-shifting effect of serotonin, suggesting that the mechanism by which serotonin mediates its effects on SCN cells involves G_i or

G_o. This is supported by preliminary experiments in which cAMP radioimmunoassays were performed on serotonin-stimulated SCN; no stimulation of cAMP levels was found.

3. Neuropeptide Y induces phase shifts in SCN near the time of greatest sensitivity to serotonin. SCN were treated with neuropeptide Y (NPY), a neurotransmitter found in projections from the intergeniculate leaflet to the SCN, using microdrop application to the same region studied in the serotonin experiments. This region, the lateral aspect of the ventral SCN, is the site at which projections from the raphe, intergeniculate leaflet and the optic nerve synapse upon SCN neurons. NPY was applied at 6 different points in the circadian cycle. The sensitive period for NPY treatment was found to be the late subjective day, from CT 4-12. Maximal phase-advances of 5 hr were induced by treatment at CT 8. These data are plotted over the data for serotonin-induced phase-shifts. This demonstrated that, while the sensitive periods are similar, they are not identical for the two transmitters.

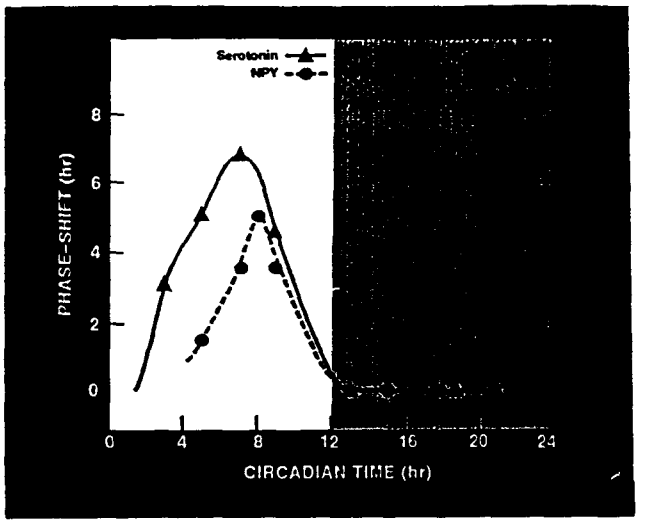


FIG. 10. COMPOSITE OF PHASE-RESPONSE CURVES FOR NPY AND SEROTONIN. THE X-AXIS DENOTE TIME OF TREATMENT AND THE Y-AXIS INDICATED THE AVERAGE MAGNITUDE OF THE PHASE-SHIFT COMPARED WITH SLICES TREATED WITH MICRODROPS OF MEDIUM. TRIANGLES = SEROTONIN; CIRCLES = NPY.

FUTURE DIRECTIONS

Over the course of the next year Marija Medanic will continue the investigate the involvement of serotonin and NPY in the mammalian circadian system. We will further investigate the signal transduction pathways by which these transmitters affect the SCN as well as their potential action on membrane excitability, assess possible interactions between serotonin and NPY, for release of both may be stimulated by dark pulses in the environment and subsequent intense activity periods, as well as determine whether serotonin induces phase-delays at other input sites to the SCN in the brain slice.

4) THE LEVELS OF GLUTAMIC ACID DECARBOXYLASE (GAD), THE BIOSYNTHETIC ENZYME FOR THE INHIBITORY NEUROTRANSMITTER GABA, AND NITRIC OXIDE SYNTHASE (NOS), A MEDIATOR OF ACTIVATION BY THE EXCITATORY AMINO ACID TRANSMITTER GLUTAMATE (GLUT), DO NOT CHANGE OVER THE CIRCADIAN CYCLE. Both excitatory and inhibitory amino acids are thought to mediate rapid neurotransmission events in the hypothalamus. Accumulating evidence from a number of sources implicates glutamate (GLUT) and GABA in excitatory and inhibitory regulation, respectively, in the SCN. We have made some initial forays into examining the role(s) if these transmitters in circadian regulation of the SCN. These include assessing activity of enzymes involved in 1) the biosynthesis of GABA and 2) a potential activation pathway of GLUT. These experiments are preliminary to study of the physiology of these two substances in the SCN.

Quantitation of Glutamic Acid Decarboxylase (GAD)

Glutamic acid decarboxylase (GAD) is the biosynthetic enzyme for the most abundant inhibitory neurotransmitter in the vertebrate brain, γ-aminobutyric acid (GABA). It has been estimated that GABA is present in 50-100% of SCN neurons (van den Pol, Moore, pers. comm.) GABA-ergic neurons, those containing GAD, are distributed throughout the nucleus, and GABA administration inhibits 65% of SCN neurons (Liou et al, 1990). GAD neurons have major synaptic inputs to vasoactive intestinal peptide containing neurons in the SCN, and are thought to play some role in light entrainment (Ralph and Menaker, 1985) as well as in gating the outflow of information from the SCN to other brain regions. Together, these results suggest that GABA plays a significant role in the physiology of SCN. The daily oscillation of endogenous SCN neuronal activity shows a 400% excursion between midday and midnight. It is likely that this may be regulated, at least in part, by changing GABA levels, which in turn would be controlled by changing GAD levels and activity over the course of the circadian cycle.

METHODS

In order to address this possibility, Dan Richard, an undergraduate distinction student, and Dr. Lia Faiman, Research Associate, undertook measuring the levels of GAD by Western blot. Two isozymes of GAD, one 65 kD and the other 67 kD, exist in the mammalian brain (Kaufman et al, 1991). We obtained antibodies specific to each form of the enzyme in order to determine the relative levels of GAD_{65} and GAD_{67}. They were used to probe SCN micropunched from brain slices that had been free-running *in vitro*. SCN were collected at four time points, circadian times (CT) 4, 10, 16, and 22, where circadian time begins with "lights on" in the rat colony and continues for 24 hr. Samples consisting of single SCN were sonicated, separated on 8% gels, blotted onto nitrocellulose, and the blots probed with primary and secondary antibodies. The Chemiluminescent Peroxidase developing system (Amersham) was used to visualize specifically bound antibody. Blots were striped and reprobed with antibody to the second isozyme. Films resulting from the chemiluminescence reaction were scanned and quantitated with an LKB densitometer. Results of quantitation were analyzed by the Tukey one-way analysis of variance and multiple range analysis at the 95% confidence level.

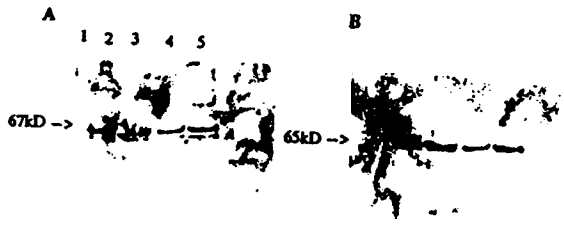
RESULTS

Both GAD isozymes show considerable fluctuation over the circadian cycle (Fig. 11, $p < 0.01$.) The GAD_{67} antibody shows significantly lower staining at CT 16 (11:00 pm) and significantly greater staining at CT 22 (5:00 am) than the points in between, CTs 4 and 10. The staining pattern for GAD_{65} is nearly identical. The lowest point in the circadian cycle is near the daily low in neuronal activity in the SCN, but a time when behavioral activity as well as activity of other circadian rhythms is high in this nocturnal species. The changing levels of GAD in the SCN seem to anticipate the transition points in the circadian day; they rise 2 hr before the entrained changes in lighting conditions. Furthermore, because these changes are taking place in the slice *in vitro*, they must be regulated by or part of the circadian system.

FUTURE DIRECTIONS

Our finding that GAD levels fluctuate in a circadian pattern in SCN *in vitro* suggests that GAD regulation may be important to circadian changes in SCN neuronal activity as well as for regulation of inputs and outputs (see page 17.) To more fully examine the relationship of GAD to the various

components of the circadian system (input-pacemaker-output), activity levels of the two GAD isozymes will be measured at time points when enzyme levels were assessed by Western blot. Additionally, the effect of GABA on the phase of the pacemaker will be determined.



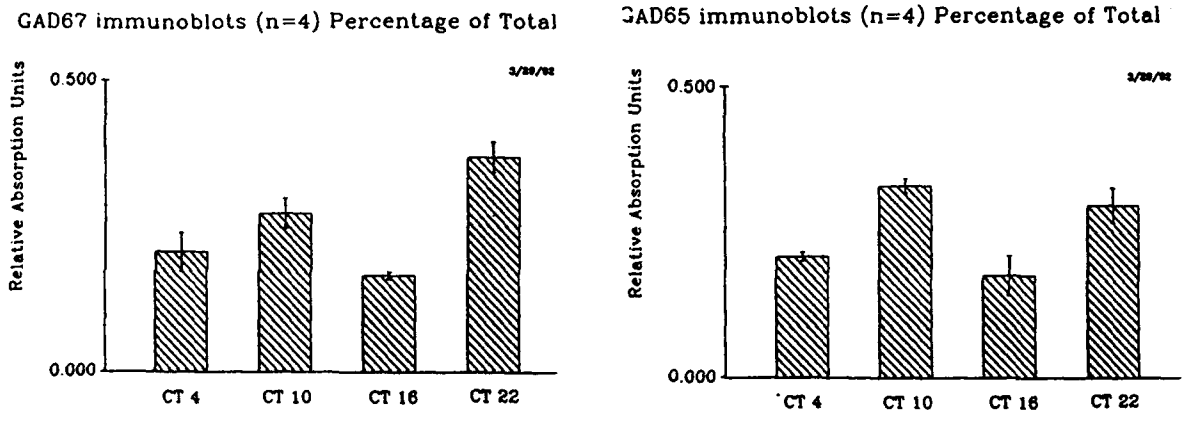


FIG. 11. GAD LEVELS CHANGE OVER THE CIRCADIAN CYCLE. TOP: IMMUNOBLOTS OF GAD_{67} AND GAD_{65} LEVELS AT 4 POINTS IN THE CIRCADIAN CYCLE. PROTEIN FROM 4 SCN COLLECTED BY MICROPUNCH FROM BRAIN SLICES AT CT 4, 10,16 OR 22 WAS LOADED ON EACH LANE OF AN 8% GEL, SEPARATED BY ELECTROPHORESIS, BLOTTED AND VISUALIZED BY THE CHEMILUMINESCENCE SYSTEM. BOTTOM: QUANTITATION OF $GAD_{67\ AND\ 65}$ FROM 4 EXPERIMENTS.

NITRIC OXIDE SYNTHASE IN THE SCN

The primary excitatory amino acid that has been implicated in the physiology of the SCN is glutamate (GLUT). GLUT is hypothesized to be the principle transmitter carrying information to the SCN from the retina via the optic nerve, although other glutamatergic inputs from regions lateral and dorsal to the SCN have recently been described (Kim and Dudek, 1992.) Investigation of release of GLUT from the optic nerve and its effect on the acute response of the SCN are described in Dr. Rea's section (#5), which follows this portion. We have made some progress relating GLUT to the SCN response by examining whether the signal transduction mechanism involves activation of nitric oxide synthase (NOS), presently known to be activated in some neurons by the GLUT-mediated rise in intracellular Ca^{++}. This work was undertaken this year by a new postdoctoral fellow, Dr. Ding. Using the histochemical reaction method based on reduction of nitro blue tetrazolium, he examined SCN for evidence of this enzyme. Our initial studies suggest that there may be a very low level of NO synthase localized to the most lateral aspect of the ventral SCN, the region to which optic nerve afferent project. We could not elevate the enzyme level with short exposures to light nor block light-induced *fos* expression with systemic injection of inhibitors of nitric oxide synthase, although prolonged exposure to light (weeks) did appear to elevate endogenous levels. Dr. Ding examined NO synthase levels in both mid night and day, times at which GLUT would have very different effects upon the SCN circadian system. This finding will be pursued in the laboratory of our collaborator, Dr. Mike Rea, with specific antibodies to NO synthase and in the brain slice where effects on the SCN can be assessed directly.

5) THE RETINOHYPOTHALAMIC TRACT AFFECTS THE SCN BY EXCITATORY AMINO ACIDS (EAAS).
This hypothesis was addressed in Dr. Mike Rea's laboratory at the USAF-SAM. The preparation he used was the horizontal slice with optic nerves attached. The ability of optic nerve stimulation to induce release of EAAs from preloaded optic nerve terminals was examined. Additionally, the potential role of GABA in day-night differences in optic nerve stimulated field potentials was studied. Finally, he has begun optical recording of the response to optic nerve stimulation in SCN preloaded with voltage sensitive dyes. The results of these experiments, provided by Dr. Rea, follow.

METHODS

A system for the study of the neurochemistry of retinohypothalamic tract (RHT) stimulation-induced electrical activity in the SCN using the horizontal hypothalamic slice was developed in year 1. The chamber, which is fabricated from Plexiglas, has a steady-state solution volume of 300 microliters and is jacketed to permit temperature control. Ports in the side of the chamber allow the introduction of suction electrodes at the level of the slice. The floor of the chamber is composed of Sylgard which provides a soft surface onto which the slice is secured with fine silver tacks. The chamber is mounted on the stage of a stereomicroscope and the slice can be observed during an experiment by transillumination.

The slice is totally submerged and constantly superfused with oxygenated Krebs-Ringer bicarbonate buffer. The buffer is delivered to and removed from the chamber by a multichannel peristaltic pump. The buffer enters the chamber at the level slice and is removed at a slightly higher rate from the top of the chamber. This arrangement maintains a constant rate of flow through the chamber, determined by the rate of buffer delivery (typically 0.8 ml/min), and results in a constant solution volume of 0.3 ml. An examination of the flow characteristics of the chamber using colored dyes demonstrated efficient slice superfusion and showed that the solution in the

chamber is completely replaced approximately every 2 minutes. At a flow rate of 0.8 ml/min and a temperature of 37°C, the partial pressure of oxygen in the chamber is 550 mm Hg.

Electrical stimulation of one optic nerve (0.7 mA square wave pulse of 300 usec duration; 1 to 5Hz) elicits a robust field potential response (160 $\pm$ 70 uV; latency = 12 $\pm$ 1 msec) in the contralateral SCN. In the rat slice, the response is most pronounced when the recording electrode is located in the ventrolateral aspect of the SCN. The field potential is totally blocked by 1 uM TTX and requires the presence of extracellular calcium. Furthermore, the field potential is reversibly blocked by selective non-NMDA glutamate receptor antagonists such as DNQX. These results demonstrate that the slice is both structurally intact and viable, and support the theory that RHT neurotransmission is mediated by excitatory amino acids.

PROGRESS

Stimulated Release of Excitatory Amino Acids

During the summer, we continued our effort to demonstrate the release of excitatory amino acids from hypothalamic slices in response to optic nerve stimulation. Unfortunately, we were unable to reproducibly evoke release of [^{3}H]-glutamate from preloaded SCN tissue. In one series of experiments, slices were collected from rats that were sacrificed at the end of the light period and used for release studies between CT16 and CT18 (mid subjective night). Here, we reasoned that release of EAA from RHT terminals may be higher at night since light exposure at night causes phase shifts of the SCN pacemaker while light during the subjective day does not. Again, optic nerve stimulation failed to consistently evoke release of [^{3}H]-glutamate from the slices. However, we observed that the response of night slices to optic nerve stimulation was different than that of day slices. Night responses tended to be somewhat larger than day responses. Furthermore, day responses saturated at stimulus intensities of about 0.7 mA, while night responses continued to increase with stimulus intensity to more than 1.5 mA. This observation may be important since it demonstrates that responses of SCN neurons to RHT stimulation are phase-dependent.

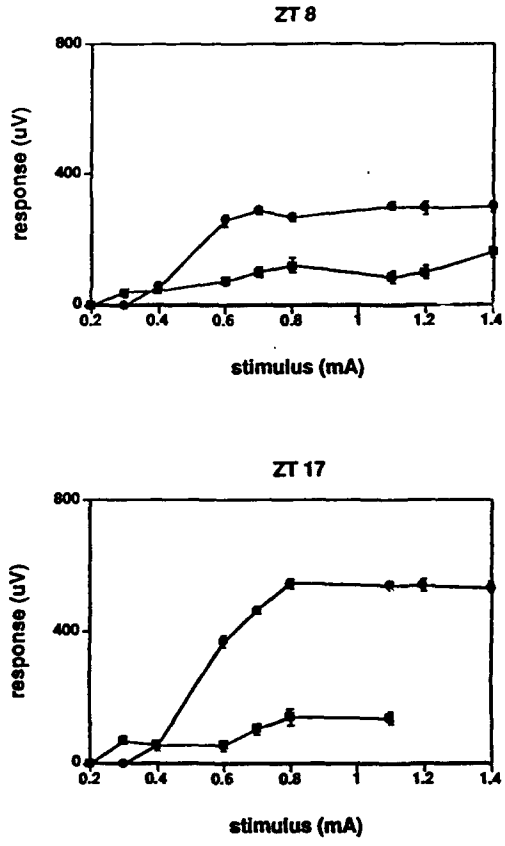
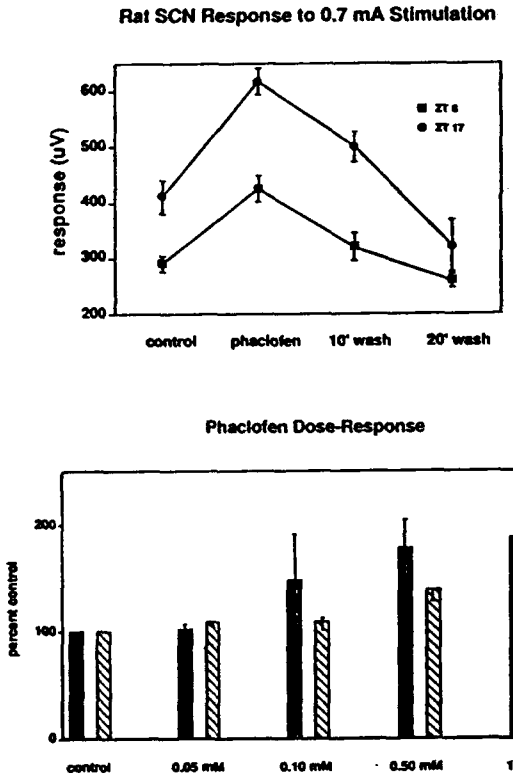


FIG. 12. STIMULUS-RESPONSE FOR HYPOTHALAMIC SLICES STIMULATED AT EITHER CT 8-9 (TOP PANEL) OR CT 17-18 (BOTTOM PANEL). DATA POINTS REPRESENT THE MEAN ± SEM OF THE AMPLITUDE (IN μVOLTS) OF THE FIELD POTENTIAL RESPONSE RECORDED FROM THE CONTRALATERAL SCN (FILLED CIRCLES), OR THE AMPLITUDE OF THE COMPOUND ACTION POTENTIAL RECORDED FROM THE STIMULATED OPTIC NERVE (FILLED SQUARES).

In the fall, we were forced to leave our laboratory at USAF-SAM for asbestos abatement. Dr Deborah Armstrong kindly offered to provide some space for our physiology setup in her laboratory at the University of Texas at San Antonio. However, restrictions on the use of radioactive compounds in Dr Armstrong's laboratory and the unavailability of an HPLC system made it impossible to conduct release studies during this period (October through May). Therefore, we elected to continue to investigate day-night differences in the field potential response to optic nerve stimulation.

SCN Slice Physiology

While at UTSA, we conducted a detailed investigation of day versus night responses in the slice. All slices were prepared during the subjective day to avoid surgically-induced phase shifts (Gillette, 1987). Slices were prepared 4 to 5 hours prior to recording and were maintained under identical conditions in superfusion chambers. Stimulus-response curves were constructed for each slice. Recording electrodes were placed in the ventrolateral aspect of the SCN contralateral to the stimulated optic nerve. Maximum field potential responses to optic nerve stimulation were consistently larger in night (CT18) versus day (CT8) slices (Fig 12). Concomitant monitoring of compound action potential trains indicated that optic nerve responses were similar in night versus day slices. Our interpretation of these results is that a larger population of SCN neurons is responsive to optic nerve stimulation in night versus day slices.



Next, we sought to determine the mechanism responsible for the augmented nocturnal response to optic nerve stimulation. Since GABA B agonists have been shown to block light-induced phase shifts of the SCN pacemaker (Ralph and Menaker, 1989), we hypothesized that the day-night difference in the response to optic nerve stimulation may be due to a rhythm in GABA tone in the SCN. Therefore, we investigated the effects of GABA B antagonists on optic nerve induced field potentials in day versus night slices. We found that the GABA B antagonist, phaclofen, dose-dependently potentiated optic nerve responses in both day and night slices (Fig 13). This important observation shows that intrinsic GABAergic activity is present in the SCN slice and is consistent with the

FIG. 13. EFFECT OF PHACLOFEN.
TOP PANEL: EFFECT OF 0.5 MM PHACLOFEN ON THE AMPLITUDE (IN μVOLTS) OF THE FIELD POTENTIAL RESPONSE RECORDED IN THE CONTRALATERAL SCN IN RESPONSE TO OPTIC NERVE STIMULATION (0.7 MA). SLICES WERE STIMULATED EITHER AT CT 8-9 (FILLED SQUARES) OR CT 17-18 (FILLED CIRCLES).
BOTTOM PANEL: DOSE RESPONSE DATA FOR PHACLOFEN ON THE AMPLITUDE OF THE FIELD POTENTIAL RESPONSE (EXPRESSED AS % OF CONTROL RESPONSE) AT STIMULATION INTENSITIES OF EITHER 0.5 MA (STIPPLED BOXES) OR 0.7 MA (CROSSHATCHED BOXES).

hypothesis that a rhythm in GABA release from SCN neurons may be responsible for "gating" the response to optic nerve stimulation among a population of neurons in the SCN. Experiments are currently underway to determine whether the release of GABA from SCN slices incubated in the presence of the GABA uptake inhibitor, nipecotic acid, is higher in day versus night slices.

Optical Recording of the SCN Slice

The results obtained from field potential measurements suggests that a population of SCN neurons may be selectively responsive to optic nerve stimulation at night. Optical recording technology using potentiometric dyes offers the potential of recording evoked responses from many SCN neurons simultaneously and could provide the spacial resolution necessary to detect regional differences in the response of SCN neurons to optic nerve input . We have investigated the feasibility of recording optic nerve-evoked potentials in the SCN using the horizonal hypothalamic slice preparation in collaboration with Dr David Senseman at the University of Texas at San Antonio. Dr Senseman has constructed an optical recording system employing a 112 element photodiode array which, together with a Zeiss optical system, currently yields a spacial resolution of approximately 80 μm (Cohen and Lescher, 1986). Custom data acquisition hardware and software permits data collection simultaneously from all 112 elements with a temporal resolution of 0.5 msec (Senseman, 1990). Using this system, we successfully recorded electrical responses of SCN neurons to optic nerve stimulation (Fig 14). The stimulus response characteristics of the optically-recorded slice were virtually identical to those observed using conventional extracellular recording techniques. Responses were calcium-dependent, tetrodotoxin-sensitive and blocked by EAA antagonists. Preliminary data on the response to GABA antagonists reveals interesting regional differences that will be the subject of future experimentation. In addition, this preparation will be exploited in an attempt to detect phase-dependent regional responses to optic nerve stimulation in the SCN.

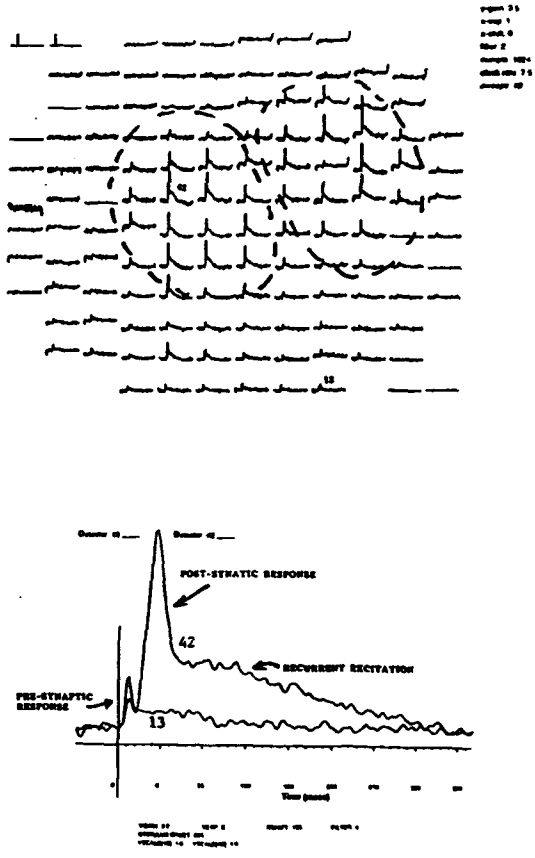


FIG. 14. OPTICAL RECORDING OF SCN RESPONSES TO OPTIC NERVE STIMULATION.
TOP PANEL: ELECTRICAL ACTIVITY IN THE HYPOTHALAMIC SLICE IN RESPONSE TO STIMULATION OF THE RIGHT OPTIC NERVE. TRACES WERE RECORDED SIMULTANEOUSLY USING A 112 ELEMENT PHOTODIODE ARRAY POSITIONED OVER THE SLICE. THE DOTTED LINES ROUGHLY REPRESENT THE BOUNDARIES OF THE SCN.
BOTTOM PANEL: HIGH RESOLUTION TRACES OF DATA RECORDED IN DETECTORS 13 (LOWER TRACE) AND 42 (UPPER TRACE) DURING THE RUN SHOWN IN THE TOP PANEL.

FUTURE APPROACHES

Stimulated Release of Excitatory Amino Acids

We returned to our laboratory in late May, and we are currently preparing to resume our study of optic nerve stimulation-induced release of radiolabeled glutamate. We have modified our recording and superfusion bath to (1) reduce the dead volume to 200 μl and (2) permit stimulation of both optic nerves simultaneously. In the next series of experiments, we will preload the slice with [^{3}H] glutamine, a precursor for releasable glutamate. Initially, we will stimulate release of [^{3}H] glutamate using 40 mM K^+ to determine the optimal conditions for optic nerve-stimulated release.

In addition, Dr Namboodiri at Georgetown University is prepared to analyze SCN superfusates for the dipeptide N-acetyl aspartyl glutamate, NAAG, the putative precursor of the GLUT used as a transynaptic messenger.

SCN Slice Physiology

Experiments to determine whether a rhythm in GABA tone in the SCN is responsible for the day-night difference in the field potential response to optic nerve stimulation are underway. In addition, we will investigate the role, if any, of GABA A receptors in this phenomenon.

Optical Recording of the SCN Slice

Dr Senseman has recently installed a new high resolution 448 element photodiode array detector in his optical recording system. This new hardware should push spacial resolution to 20 μm. This will place up to 90 elements over each SCN and should provide a revealing high resolution image of optic nerve evoked electrical activity in the SCN. We will use this system to search for phase-dependent patterns (1) of electrical activity in response to optic nerve stimulation, and (2) in the pharmacological sensitivity of evoked responses in the rodent SCN.

Role of Cyclic Nucleotides in RHT Neurotransmission

This summer, Todd Weber will visit the Armstrong Laboratory to conduct a series of experiments designed to determine whether light-induced phase shifts of SCN-driven rhythms involve the activation of cyclic nucleotide generating systems in the SCN. Todd is an Air Force Graduate Fellow currently working toward his PhD in Dr Gillette's laboratory. Specifically, Todd will determine whether (1) light exposure during the subjective night causes accumulation of cGMP in the SCN, (2) administration of cGMP analogs into the region of the SCN causes light-like phase shifts of the free-running activity rhythm in hamsters, (3) administration of cGMP analogs induces a light-like pattern of c-fos expression in the SCN, and (4) cGMP antagonists and nitric oxide synthase inhibitors attenuate light-induced phase shifts. These studies will employ the collaborative efforts of several investigators at the Armstrong Laboratory, including Dr Lewis Lutton, Dr Paul Werchan, and Dr Robert Gannon.

RESEARCH ARTICLES PUBLISHED OR PLANNED FOR TECHNICAL JOURNALS

Medanic, M. and Gillette, M.U. 1992. Serotonin regulates the phase of the rat suprachiasmatic circadian pacemaker *in vitro* only during the subjective day. *Journal of Physiology* **450:** 629-642. (This is appearing in the May issue; reprints have not yet arrived.)

Gallman, E.A. and Gillette, M.U. 1992. Diversity in neurons of the suprachiasmatic nucleus (SCN) studied by whole cell recording in rat brain slice. In preparation for summer submission. *Journal of Physiology.*

Tcheng, T.K., McArthur, A.J., Liu, C., Medanic, M. and Gillette, M.U. 1992. Localization of the circadian pacemaker in the suprachiasmatic nucleus brain slice and its intrinsic organization. *Journal of Neuroscience.* This will be prepared for publication within the next six months.

PARTICIPATING PROFESSIONALS

Martha U. Gillette, P.I., Associate Professor of Cell & Structural Biology, and of Physiology. College of Liberal Arts and Sciences, and of Medicine. Beckman Research Institute Affiliate. University of Illinois at Urbana-Champaign.

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Eve A. Gallman, Postdoctoral Research Associate, Department of Cell & Structural Biology; University of Illinois

Marija Medanic, Systems and Integrative Training Grant Trainee and Graduate Research Assistant, Department of Physiology & Biophysics, University of Illinois; M.S. awarded in Jan., 1991; Pursuing Ph.D. currently.

Ann-Marie Michel, Research Specialist in Biological Science; working at USAF School of Aerospace Medicine; appointed through the University of Illinois

Daniel Richard, Undergraduate Distinction Student, Department of Cell & Structural Biology, University of Illinois

Thomas K. Tcheng, Graduate Research Assistant, Neuroscience Program, University of Illinois; currently taking Qualifying Examination for the Ph.D. program.

Todd Weber, Air Force Graduate Fellow, pursuing Ph.D. in Physiology & Biophysics, University of Illinois.

INTERACTIONS THROUGH MEETINGS AND COLLABORATIVE EXPERIMENTS

MEETINGS

Gillette, M.U. 1991. Analysis of phase-locked regulators of circadian rhythms in the SCN brain slice. Invited lecture presented to the Gordon Conference on Chronobiology, Irsee, Germany, October, 1991.

Tcheng, T.K. and Gillette, M.U. 1991. Characterization of regional neuronal activity in the suprachiasmatic nucleus using a curve-fitting technique. Presented at the Society for Neuroscience Meeting, November, 1991, New Orleans, LA

Gallman, E.A., Nolan, P.C., Waldrop, T.G. and Gillette, M.U. 1991. Whole cell recording of neurons of the suprachiasmatic nucleus (SCN) studied in rat brain slice. Presented at the Society for Neuroscience Meeting, November, 1991, New Orleans, LA

Medanic, M. and Gillette, M.U. 1991. Serotonin agonists advance the circadian rhythm of neuronal activity in rat SCN *in vitro*. Presented at the Society for Neuroscience Meeting, November, 1991, New Orleans, LA

Richard, D., Faiman, L. and Gillette, M.U. 1991. Quantitation of glutamic acid decarboxymase (GAD) and tyrosine hydroxylase (TH) in single suprachiasmatic nuclei (SCN) across the circadian cycle. Presented at the Society for Neuroscience Meeting, November, 1991, New Orleans, LA

Gillette, M.U. 1991. Aminergic and amino acid transmitters in the control of SCN rhythms. Invited lecture presented at the Conference Philippe Laudat "Neurobiology of circadian and seasonal rhythms: animal and clinical studies", November, 1991, Strasbourg, France

Gillette, M.U., McArthur, A.J., Liu, C. and Medanic, M. 1992. Intrinsic organization of the SCN circadian pacemaker studied by long-term electrical recording *in vitro*. Presented at the Society for Research on Biological Rhythms Meeting, May, 1992, Amelia Island, FL

Satinoff, E., Li, H., McArthur, A.J., Medanic, M., Tcheng, T., and Gillette, M.U. 1992. Does the SCN oscillate in old rats as it does in young ones? Presented at the Society for Research on Biological Rhythms Meeting, May, 1992, Amelia Island, FL

Gallman, E.A. and Gillette, M.U. 1992. Whole cell recording of neurons of the suprachiasmatic nucleus (SCN) studied in rat brain slice. Presented at the Society for Research on Biological Rhythms Meeting, May, 1992, Amelia Island, FL

Medanic, M. and Gillette, M.U. 1992. NPY phase-shifts the circadian rhythm of neuronal activity in the rat SCN *in vitro*. Presented at the Society for Research on Biological Rhythms Meeting, May, 1992, Amelia Island, FL

COLLABORATIONS

With Dr. Mike Rea (USAF-SAM, Brooks AFB) we have explored a number of potential collaborative experiments. In the current year, these have been oriented around the possibility that light induced phase-shifts might be mediated through glutamate stimulation of nitric oxide synthase, followed by activation of guanyl cyclase. These should lead to induction of c-fos expression in the SCN. Todd Weber, an Air Force Graduate Fellow in my lab, has made a number of attempts to measure cGMP levels stimulated by glutamate in cerebellum, the tissue in which this pathway was first described (Bredt and Snyder, 1991.) These have produced highly variable results; since Todd is extremely experienced in this technique, we have put the project on hold while we try to implicate NO synthase more directly in light-induced phase-shifts. Todd will be spending 3 months in Dr. Mike Rea's lab at USAF-SAM this summer as part of his fellowship training. There Todd will continue experiments related to his dissertation research directed at elucidating the signal transduction pathway by which excitatory amino acids affect the SCN. Dr. Rea has described the experiments that Todd will undertake with his group this summer on page 19 in his section. Additionally, Mike and I have discussed Ding's finding of very low staining in SCN for NO synthase; Mike will continue to localize this enzyme in the SCN with antibodies and we will continue to try to prove physiologically whether or not it is important to nighttime phase-shifting.

I value the intelluctual company of several members of the group funded by AFOSR including Mike Rea, Ed Dudek and David Earnest. They are ever available for consultation or discussion of methods and results in these studies.

SUMMARY OF PROGRESS

1) The preponderance of data suggest that the SCN pacemaker is relatively distributed and is organized primarily in the VL-SCN, the region receiving afferent fibers from regulatory brain regions.

2) Neurons of the SCN are not homogeneous, but rather represent a diverse population with a range of electrophysiological characteristics. Based on other descriptive studies of pacemaker neurons in other brain regions, some of these characteristics might be expected to be found in oscillatory neurons.

3) Serotonin and NPY are both potent regulators of the SCN when briefly and focally applied. They induce phase-advances during the daytime portion of the circadian cycle only; at nighttime they are without effect when applied focally to the site that raphe afferents terminate. Serotonin appears to act through a $5HT^{1A}$ receptor via a pertussis-sensitive G protein. Half maximal responses are achieved at 10^{-9} M.

4) Glutamic acid decarboxylase (GAD) levels undergo significant diurnal variation over the circadian cycle. Initial experiments suggest it unlikely that nitric oxide synthase links glutamate receptor stimulation to enhanced cGMP levels.

5) Evidence that glutamate is the transmitter in the optic nerve at the SCN is growing. Furthermore, GABA may play a role in gating optic nerve stimulation at night.

Journal of Physiology (1992), ***, pp. ***-***
With 5 figures
Printed in Great Britain

SEROTONIN REGULATES THE PHASE OF THE RAT SUPRACHIASMATIC CIRCADIAN PACEMAKER *IN VITRO* ONLY DURING THE SUBJECTIVE DAY

BY MARIJA MEDANIC AND MARTHA U. GILLETTE*†

*From the Department of Physiology and Biophysics, University of Illinois, 407 S. Goodwin Ave., Urbana, IL 61801, USA and the * Departments of Cell Structural Biology, Physiology and Biophysics and The Neuroscience Program, University of Illinois, 505 S. Goodwin Ave., Urbana, IL 61801, USA*

(*Received 1 August 1991*)

SUMMARY

1. The suprachiasmatic nucleus (SCN) of the hypothalamus is the primary pacemaker for circadian rhythms in mammals. The 24 h pacemaker is endogenous to the SCN and persists for multiple cycles in the suprachiasmatic brain slice.

2. While serotonin is not endogenous to the SCN, a major midbrain hypothalamic afferent pathway is serotonergic. Within this tract the dorsal raphe nucleus sends direct projections to the ventrolateral portions of the SCN. We investigated a possible regulatory role for serotonin in the mammalian circadian system by examining its effect, when applied at projection sites, on the circadian rhythm of neuronal activity in rat SCN *in vitro*.

3. Eight-week-old male rats from our inbred colony, housed on a 12 h light: 2 h dark schedule, were used. Hypothalamic brain slices containing the paired SCN were prepared in the day and maintained in glucose and bicarbonate-supplemented balanced salt solution for up to 53 h.

4. A 10^{-11} ml drop of 10^{-6} M-serotonin (5-hydroxytryptamine (5-HT) creatinine sulphate complex) in medium was applied to the ventrolateral portion of one of the SCN for 5 min on the first day *in vitro*. The effect of the treatment at each of seven time points across the circadian cycle was examined. The rhythm of spontaneous neuronal activity as recorded extracellularly on the second and third days *in vitro*. Phase shifts were determined by comparing the time-of-peak of neuronal activity in serotonin- *vs.* media-treated slices.

5. Application of serotonin during the subjective day induced significant advances in the phase of the electrical activity rhythm ($n = 11$). The most sensitive time of treatment was CT 7 (circadian time 7 h after 'lights on' in the animal colony), when a $7{\cdot}0 \pm 0{\cdot}1$ h phase advance was observed ($n = 3$). This phase advance was perpetuated on day 3 *in vitro* without decrement. Serotonin treatment during the subjective night had no effect on the timing of the electrical activity rhythm ($n = 9$).

6. The specificity of the serotonin-induced phase change was assessed by treating

† To whom correspondence should be addressed.

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CHARACTERIZATION OF REGIONAL NEURONAL ACTIVITY IN THE SUPRACHIASMATIC NUCLEUS USING A CURVE-FITTING TECHNIQUE

T.K. Tcheng and M.U. Gillette. Neuroscience Program and Department of Cell & Structural Biology, University of Illinois, Urbana, IL 61801.

The rat suprachiasmatic nuclei (SCN) contain an endogenous circadian pacemaker that is expressed in the brain slice as a 24 hr oscillation in ensemble neuronal firing rate (ENFR). We are studying the anatomical distribution of the pacemaker within the SCN by analyzing the ENFR in isolated dorsomedial (DM) and ventrolateral (VL) subregions.

We have previously demonstrated empirical changes in the ENFR of the DM-SCN after surgical isolation. Our current work quantifies these changes and characterizes neuronal activity in the isolated regions. Single unit activities were recorded over >24 hr to examine the circadian pattern of ENFR for each region. A parameterized curve was constructed that describes the 24 hr ENFR of control SCN in an intact slice. Goodness-of-fit for ENFRs from isolated DM- and VL-SCN were calculated after fitting the curve to 24 hr data from control SCN. Significant differences ($p < 0.05$) were found between controls (N=4) and isolated DM-SCN (N=2) but not isolated VL-SCN (N=3). Spectral analysis will be performed on control and experimental data to identify the major periodicities expressed by each group. (Supported by AFOSR grant 90-0205.)

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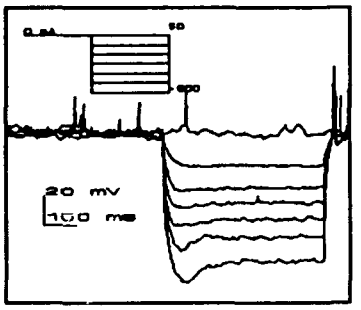
WHOLE CELL RECORDING OF NEURONS OF THE SUPRACHIASMATIC NUCLEI (SCN) STUDIED IN RAT BRAIN SLICE.

E.A. Gallman, P.C. Nolan*, T.G. Waldrop and M.U. Gillette. Depts of Cell & Structural Biology and Physiology & Biophysics, University of Illinois, Urbana, IL, 61801.

The SCN in rat are the neuroanatomic substrate for the biological pacemaker underlying circadian rhythmicity. This rhythmicity is expressed *in vitro* in the hypothalamic brain slice preparation as a daily oscillation in overall firing frequency of SCN neurons, with peak activity occurring near the middle of subjective day. The intracellular mechanisms responsible for changes in firing frequency are unknown. As these cells are small (6-15 μm), they have proved hard to study with conventional intracellular techniques, and few such studies exist. We have begun examining these cells using whole cell patch recording in slice. Typically, these cells had resting potentials -40 to -60 mV, after-hyperpolarization (AHP) -5 to -15 mV, and overshooting action potentials of 55 to 85 mV amplitude. Our results also indicate that at least some SCN neurons have an inward rectifier current and many exhibit post-inhibitory rebound (PIR).



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SEROTONERGIC AGONISTS ADVANCE THE CIRCADIAN RHYTHM OF NEURONAL ACTIVITY IN RAT SCN IN VITRO.

M. Medanic and M.U. Gillette, Dept. of Physiol. & Biophys. and Dept. of Cell & Struct. Biol., Univ. of Illinois, Urbana, IL 61801.

Serotonin (5-HT) directly affects the SCN pacemaker *in vitro*. Brief application of 5-HT to ventrolateral (VL) SCN during the subjective day phase-advances (ϕ_A) the time of peak neuronal activity with a maximal shift of 6.9±0.1 hr at CT 7. 5-HT is ineffective at night. This temporal sensitivity matches that for cAMP analogs. To confirm the specificity of 5-HT-induced ϕ_A and to investigate the mechanism by which 5-HT acts on the SCN, the effects of two 5-HT_1 agonists, 5-CT and 8-OH DPAT, were tested.

Hypothalamic brain slices containing the paired SCN were obtained from male Long-Evans rats (8wks old, raised in 12L:12D) and maintained *in vitro*. The slices were treated with 10^{-6}M 5-CT at CT 9 (n=3) or 15 (n=2), or with 10^{-6}M 8-OH DPAT at CT 9 (n=3) on day 1, by a 30 *f*l drop to the VL-SCN for 5 minutes. The time-of-peak in the rhythm of neuronal activity was accessed the following day.

While treatment of the VL-SCN with 5-CT at CT 15 did not significantly alter the rhythm, exposure at CT 9 resulted in a 6.0±0.1 ϕ_A of the time-of-peak. Similarly, administration of 8-OH DPAT at CT 9 induced a 6.9±0.1 ϕ_A of the peak time. This confirms the specificity of the 5-HT-induced ϕ_A and lends support to the hypothesis that 5-HT may affect the SCN via a 5-HT_1 receptor-linked pathway. (Supported by AFORS grant 90-0205.)

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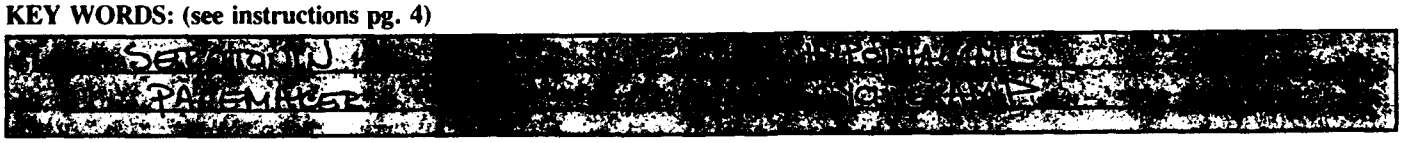

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QUANTITATION OF GLUTAMIC ACID DECARBOXYLASE (GAD) AND TYROSINE HYDROXYLASE (TH) IN SINGLE SUPRACHIASMATIC NUCLEI (SCN) ACROSS THE CIRCADIAN CYCLE.

D. Richard*, L. Faiman & M.U. Gillette. Dept. of Cell & Struct. Biol., Univ. of Illinois, Urbana, IL 61801

To elucidate the organization of substrates underlying the circadian pacemaker, we are quantitating identifiable proteins in single SCN. The SCN are derived from hypothalamic slices of rat brain maintained in glucose/$NaHCO_3$-supplemented minimal salts, conditions under which the SCN continue the circadian rhythm of neuronal firing observed *in vivo*. At a desired timepoint and after a fixed incubation period, a slice is quick-frozen on dry ice and one SCN (~5 μg protein) punched out. Proteins are separated by SDS-PAGE and identified immunologically by Western blot.

Using this technique, we determined levels of GAD and TH, the biosynthetic enzymes for GABA and dopamine, respectively, throughout the circadian cycle. Since TH is a protein kinase A substrate, we titrated phosphorylatable sites by cAMP-stimulated *in vitro* phosphorylation in the same samples. The level of TH relative to the degree of PKA phosphorylation was compared directly to assess the endogenous TH phosphorylation state.

Levels of GAD and TH were studied to develop the parameters for quantifying other regulatory proteins and their phosphorylation states in single SCN over the circadian cycle.

KEY WORDS: (see instructions pg. 4)

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2. PROTEIN KINASE A
3. GABA
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INTRINSIC ORGANIZATION OF THE SCN CIRCADIAN PACEMAKER STUDIED BY LONG-TERM ELECTRICAL RECORDING IN VITRO.

M.U. Gillette, A.J. McArthur, C. Liu and M. Medanic. Depts. of Cell & Structural Biology, Physiology & Biophysics and The Neuroscience Program, University of Illinois, Urbana, IL 61801.

The suprachiasmatic nuclei (SCN) contain a circadian clock that expresses a near 24-hr rhythm in the ensemble neuronal firing rate in rats. This pattern persists in brain slice preparations and is highly reproducible between experiments. Spectral analysis of the electrical activity rhythm in the major anatomical subdivision, the ventrolateral (VL) and dorsomedial (DM) SCN, indicates that both express the characteristic pattern seen in integrated analysis of the SCN rhythm (Tcheng,& Gillette, 1989.) Furthermore, this pattern persists in both regions *in vitro* when the contralateral fibers between the paired SCN are cut by bisection of the optic chiasm. Hemisection of each SCN in the bisected slice, which surgically isolates the DM from the VL region, results in disparate changes in each region's firing pattern: a robust rhythm persisted in the VL region but was less evident in the isolated DM region (Tcheng & Gillette, 1990).

We have examined these electrical activity patterns in subdivided SCN in greater detail by monitoring the ensemble firing pattern over 30 hr *in vitro*. Most experiments comprised continuous records from a single SCN. Preparation and dissection of all brain slices were done near CT 1. Extracellular monitoring of SCN neurons were sampled continuously through day 2 *in vitro*. The data reveal that the neurons of the isolated VL-SCN generate a robust circadian pattern of activity that is indistinguishable from the rhythm of SCN-intact slices. However, the isolated DM region shows an impaired rhythm both in form and amplitude (n=3). While some rhythmicity may persist, activity is generally damped to $< 1/2$ that seen in isolated VL-SCN and is unusual in the paucity of high frequency units. Interestingly, biasing the bisecting cut toward the VL region can restore the high amplitude rhythm. These results are consistent with our earlier suggestion that a robust pacemaker is located in the VL-SCN region, that extent to the center of the nucleus. We cannot conclude that the DM region is without a pacemaker. Yet, should it exist, it must be more labile. Supported by AFOSR grant 90-0205.

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WHOLE CELL RECORDING OF NEURONS OF THE SUPRACHIASMATIC NUCLEUS (SCN) STUDIED IN RAT BRAIN SLICE.

Eve A. Gallman and Martha U. Gillette. Depts. of Cell & Structural Biology and Physiology & Biophysics, Univ. of Illinois, Urbana, IL, 61801.

The SCN are a neuroanatomic substrate for the mammalian circadian pacemaker. The mean firing rate of SCN neurons varies in a circadian pattern. This neural correlate of circadian rhythm is retained in the rat hypothalamic brain slice. Our laboratory is investigating the roles of signal transduction and second messenger systems in the ability of the SCN to maintain 24 hour time *in vitro*. To this end, we are employing the whole cell patch technique, in rat brain slice, to examine the intrinsic membrane properties of SCN neurons across the circadian cycle.

We have studied over 50 neurons within the ventral SCN, characterizing 4-12 neurons per 4 hour time bin around the clock. Many cells (75%) were spontaneously active. Both regular firing and irregular bursting patterns were noted, and one neuron produced a regular and robust burst. While only 2 of 10 cells recorded at CT 8-12 were silent, 6 of 12 recorded at CT 12-16 were silent. The biggest change in mean resting membrane potential occurred predusk (-51.1 mV $\pm$ 1.7,CT 4-8; -55.7 mV $\pm$ 1.7, CT 8-12). Of 5 cells tested at CT 8-12, only 1 exhibited spike frequency adaptation (SFA); however, 12 of 13 cells across the remaining 5 time bins showed SFA. All neurons exhibiting SFA showed afterhyperpolarization following a depolarizing pulse. Depolarizing 'sag' was seen during hyperpolarization in 67% of cells tested. Most neurons (80%) exhibited post-inhibitory rebound firing.

We conclude, from the overall data, that the ventral SCN is populated by a variety of electrophysiologically distinct cell types. Futhermore, the data concerning parameters measured across the circadian cycle, while very preliminary, suggest that the period just prior to dusk may be of particular importance.

AFOSR 90-0205; PHS NS 22155 & NRSA NS 09155.

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NPY PHASE-SHIFTS THE CIRCADIAN RHYTHM OF NEURONAL ACTIVITY IN THE RAT SCN *IN VITRO*

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The geniculo-hypothalamic tract carries visual information from the lateral geniculate nucleus to the suprachiasmatic nuclei (SCN). The axons of this tract are immunoreactive for neuropeptide Y (NPY) and their terminals overlap with retinal and serotonergic inputs to the SCN (Card & Moore, 1984; Guy *et al.* 1987). NPY microinjected into the SCN region is a strong phase-shifting agent of behavioral rhythms in hamsters causing phase advances in the daytime (peaking CT 4-6, Albers & Ferris, 1984). We are investigating the role of NPY in the mammalian circadian system in greater detail by examining its effect on the rhythm of neuronal activity in the SCN *in vitro*, when administered directly to the SCN at projection sites from the geniculate neurons.

Male Long-Evans rats were raised for 8 weeks on 12L:12D in our in bred colony. 500μm coronal slices containing the paired SCN were prepared during the daytime, and maintained for 2 days in glucose and bicarbonate supplemented minimal salts solution. The ventrolateral region of one of the SCN in the slice was treated with a 30 μl drop of 10^{-6}M NPY on day 1 *in vitro* at different time points across the circadian cycle. The effects of NPY applied at CT 5 (n=2), 7 (n=3), 9 (n=2) and 15 (n=2) were assessed by recording extracellularly the time-of-peak in the rhythm of electrical activity on day 2 *in vitro*.

Exposure of the SCN to NPY during the daytime resulted in large phase shifts in the time-of-peak, with the highest sensitivity between CT 7-9 (3.5 h phase-advance). Treatment at CT 15 did not induce an observable phase-shift in the electrical activity rhythm. The time of sensitivity to phase-shifting of rats by NPY microdrops *in vitro* is slightly later then the peak sensitivity of hamsters when NPY was injected into the SCN region *in vivo*. Interestingly, the point of greatest phase-shifting effect of NPY occurs at a time when rat SCN are also highly susceptible to phase-shifting by serotonin (Medanic & Gillette, 1992).

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